

1 **Lab-on-chip clinorotation system for live-cell microscopy under** 2 **simulated microgravity**

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Abstract

Cells in microgravity are subject to mechanical unloading and changes to the surrounding chemical environment. How these factors jointly influence cellular function is not well understood. We can investigate their role using ground-based analogues to spaceflight, where mechanical unloading is simulated through the time-averaged nullification of gravity. The prevailing method for cellular microgravity simulation is to use fluid-filled containers called clinostats. However, conventional clinostats are not designed for temporally tracking cell response, nor are they able to establish dynamic fluid environments. To address these needs, we developed a Clinorotation Time-lapse Microscopy (CTM) system that accommodates lab-on-chip cell culture devices for visualizing time-dependent alterations to cellular behavior. For the purpose of demonstrating CTM, we present preliminary results showing time-dependent differences in cell area between human mesenchymal stem cells (hMSCs) under modeled microgravity and normal gravity.

Keywords: space biology; clinorotation; clinostat; live-cell; microscopy; stem cell

1. Introduction

Cellular specimens in spaceflight exhibit abnormal, time-evolving morphology and cytoarchitecture (e.g. cytoskeleton, focal adhesions, etc.), which may affect certain cell events including replication, differentiation, migration, and signaling [1-3]. These events generally confer broader changes to tissues that can lead to reduced bone mineral density [4,5], muscle atrophy [6,7], back pain [8,9], and other ailments [10]. The success of long-duration human space exploration requires countermeasures that address the fundamental cellular changes

adopted in microgravity and are most effective if they consider the underlying dynamic processes driving these alterations.

The National Aeronautics and Space Administration (NASA), European Space Agency (ESA), and other organizations manage a robust portfolio of research initiatives for space biology, using the International Space Station (ISS) as their flagship facility. However, the ISS is not easily accessible and does not often accommodate continuous monitoring of onboard experiments, thereby limiting the ability to observe time-evolving processes. While ground-based microgravity simulations with conventional clinostats [11-13] are notably less expensive, they also preclude the possibility of real-time cell monitoring. State-of-the-art methods do not easily allow time-dependent investigations to identify the mechanisms of cellular alterations and may consequently lead to an incomplete understanding of how microgravity affects human health.

A brute-force remedy for this latent need is to incorporate a full-scale microscope onto a mega-scale clinorotation platform for ground simulations. Clinorotation was initially developed for studying how plants respond to gravity and is currently the prevailing method for cellular microgravity simulation. It is based on the assumption that a time-averaged nullification of gravity can be achieved by reorienting the gravity vector on biological samples, and that the reorientation is fast enough to ensure that specimens cannot perceive a gravitational bias in any direction. The ESA's clinostat microscope [14] is an example of one mega-scale configuration. Another example was published in 2010 by Pache et. al. [15] and was optimized in 2012 by Toy et. al. [16] to demonstrate how digital holographic microscopy (DHM) with mega-scale clinorotation can monitor cytoskeletal changes in simulated microgravity. Interestingly, these studies showed the first published, same-cell images exhibiting time-dependent lamellipodium

retraction, filopodia extension, and perinuclear actin accumulation under clinorotation compared to static controls.

Even though the clinostat microscope and CR-DHM can be used for time-lapse microscopy, many labs do not have the resources or facility space to incorporate a mega-scale system. Furthermore, mega-scale systems could induce significant mechanical vibrations or impulse loads that may disturb cell cultures. Therefore, we present a clinochip system for Clinorotation Time-lapse Microscopy (CTM) that may also enable long-term, low shear cell culture. While the underlying principles of the clinochip are identical to conventional clinostats, and certainly similar to the mega-scale systems, CTM enables live-cell imaging, without prohibitively large equipment or disruption of culture environments. Importantly, CTM represents a significant step forward in space biology research because it is an affordable, size-manageable system that enables microgravity studies of not only traditional endpoint outcomes, but also dynamic cellular processes.

Moreover, CTM is compatible with any lab-on-chip device assembled on a standard microscope slide, for example: microcavities for cell culture; chemical gradient generators; cell sorters; and capillary-based separation columns. It can accommodate cells in monolayer, suspension, and 3D constructs. State-of-the-art microfluidic techniques allow us to precisely modulate microscale flow to create complex cell culture environments, a feature that is not always possible with conventional clinostat devices. Specifically, the surge in microfluidics research in the past decade has enabled exciting new capabilities for probing cells in a variety of ways. This technology can easily be leveraged with CTM.

Media exchange between an external reservoir and a rotating “clinochip” platform on CTM is feasible by integrating lab-on-chips with a miniature rotary union for programmable

media exchange, continuous media circulation, and chemical infusions. Taken together, the enormous scope of possible microgravity investigations distinguishes clinochips from conventional clinostats. We believe that their affordability, easy implementation, and amenability for live-cell imaging will fully-enable researchers seeking to understand the time-evolution of cellular alterations under microgravity simulation.

2. Material and methods

2.1. CTM system

We fabricated a clinochip system that enables imaging of cells subjected to two-dimensional microgravity simulation and can be operated in parallel with a static chip as a control. The CTM configuration depicted in Fig. 1a uses a stepper motor with a resolution of 200 macrosteps per revolution and a two-gear train assembly to transfer rotational motion to a clinochip platform that holds a lab-on-chip device. This rotating platform pivots on a custom-built miniature polytetrafluoroethylene (PTFE) rotary joint that allows one rotational degree of freedom about the spin axis. Additionally, the rotary joint is equipped to manage fluid exchange between external fluid reservoirs and devices on the rotating clinochip platform.

In brief (refer to Fig. 1b), the rotary joint was fabricated with 19-gauge blunt syringe needle tips that were press-fitted from the rear of CNC-milled PTFE connectors into 1 mm access holes until flush with the microchannel groves on the front. Axially self-aligning neodymium ring magnets (RC86, K&J Magnetics) were pressed into slots at the rear of the connectors and provide substantial clamping force when mating two identical connectors. Commonly used as a material for gaskets, PTFE has some unique properties that also make it suitable for the rotary joint: 1) high compressibility forms a tighter seal at the mating interface;

2) hydrophobicity helps to prevent fluid wetting and leakage at the interface; 3) low coefficient of friction allows for easy rotation about the spin axis.

Open-loop control is established with LabVIEW (v.10.0, National Instruments) for the stepper motor (HT11-013D, Applied Motion Products), inverted fluorescence microscope (IX81, Olympus Corporation), XY motorized stage (MS-2000, Applied Scientific Instrumentation), and B/W CCD digital camera (ORCA-ER, Hamamatsu Photonics).

2.2. Lab-on-chip devices

Live-cell CTM devices were fabricated using a high-frequency corona treater (BD-20AC, Electrotech Products) to energetically bond layers of polydimethylsiloxane (Sylgard 184, Dow Corning), i.e. PDMS, at 10:1 ratio of base to curing agent, between 75x25x1 mm glass slides. Geometric features in PDMS were formed by a high-resolution razor cutter (FC8000, Graphtec). To prepare microfluidic devices for experiments, cell culture surfaces, consisting of a 200 micron tall by 1 mm wide microchannel constructed from PDMS and glass, were cleaned with 70% ethanol, rinsed in deionized water, and air-dried. Immediately before cell experiments, the microchannel was incubated in ambient for one hour with 15 ug/mL fibronectin (354008, BD Sciences) in phosphate buffer saline (PBS) without Ca^{++} and Mg^{++} and then gently rinsed 2-3 times with PBS. Fibronectin-treated surfaces were kept hydrated by filling culture cavities with fresh PBS and were sterilized by ultraviolet exposure for 15 minutes prior to cell seeding.

2.3. Cell culture experiments

Passage-5 hMSCs were expanded in 6-well plates with hMSC media until confluent. Stem cells were trypsinized, centrifuged, resuspended at 10^5 cells/mL, plated into microchannels,

and incubated in a microscope-amenable environmental chamber (Precision Plastics) at 37 °C, 50% humidity, and 5% CO₂ for 20 min before microchannels were gently flushed with hMSC media to remove non-adherent cells. One clinochip and one static chip were placed onto the CTM system, which was mounted to an XY motorized stage (MS-2000, Applied Scientific Instrumentation) on an inverted fluorescence microscope (IX81, Olympus Corporation).

A group of cells that had been seeded on both the clino- and static chip were randomly selected for time-lapse microscopy using differential interference contrast (DIC) and phase contrast. Both chips had similar seeding densities, roughly 5-6 cells in the field of view using a 10X objective, and similar initial morphologies. Before we subjected the clinochip to 60 RPM clinorotation, we acquired an initial image of both chips at 0 hrs. At each subsequent hour, for 8 hrs, we acquired additional images. Figure 2 shows same-cell images at 0, 1, 4, and 8 hr time points for 60 and 0 RPM.

Results

From these timelapse images, we measured time-evolving, same-cell areas using a custom Matlab algorithm (see Fig 3). Average areas were not different in the first 3 hrs of clinorotation. After 5 hrs however, cell areas at 0 RPM increased dramatically while cells at 60 RPM showed little change. Significant differences were found at 6-8 hr time points. Moreover, at each time point, we conducted a visual inspection of other cell groups and found that morphologies for the randomly selected cells were qualitatively representative of the entire population in the chip. Although our sample size was small, our preliminary CTM results demonstrate evidence of substantial changes to hMSC morphology that may affect other functions important to bone health including differentiation and chemotactic homing.

We also took measurements for the absolute difference of same-cell areas between each time point and the previous point, as shown in Fig. 4. While much variability exists in the data, specimens at 0 RPM were measured at approximately 70% higher average difference when compared with 60 RPM.

Discussion

The goal of this paper was to present a way to improve on state-of-the-art clinorotation devices. Since particle physics in conventional clinostats is impossible to accurately control in experiments, cells can be subjected to mechanical forces and chemical gradients that might not be physiological. Additionally, adherent cells in these clinostats need to be seeded on microcarrier beads that have limited surface area for proliferation, which prohibits long-term culture. Moreover, the constant movement of cells through culture media makes dynamic bioassays, which are important for a more holistic understanding of cellular response, generally unattainable. Finally, without the ability to manipulate culture conditions, for example, by modulating the chemical microenvironment, conventional clinostats can only offer a narrow range of possible science investigations.

In conjunction with lab-on-chip technologies, the CTM methods described in this paper addresses these issues and may enable a wide range of live-cell, time-dependent investigations in simulated microgravity. As a whole, CTM allowed us to identify the time-evolution of cell response in simulated microgravity without the limitation of only being able to obtain images at static time-points that are usually the extent of the capabilities afforded by conventional clinostat devices. Using static time points would limit the ability to understand how the time-dosage of microgravity affects cells, introduces more variability in experimental data, and may require

more experimental controls to rule out confounding factors than our CTM system. For these reasons, and for its affordability and versatility, we believe that CTM represents a significant step forward in space biology research.

Our preliminary experiments examine early spreading in hMSCs, when cells are only loosely attached and could mimic how daughter cells in mitosis may behave in microgravity. We hypothesize that microgravity-induced morphological alterations may also affect lineage commitment and may be responsible for the markedly lower rates of differentiation observed in stem cells flown in space [17]. This hypothesis warrants further study, but agrees with previously published work showing that simulated microgravity disrupts hMSC function by enhancing adipogenesis and reducing osteoblastogenesis [18,19]. In future work, we will use CTM to understand how microgravity may affect early attachment by fluorescently tagging cytoskeletal elements and correlating cell morphology with long-term rates of proliferation.

Studying hMSCs is particularly useful because they are important for maintaining bone health and play an integral role in bone fracture healing. Normal cell functions are hypothesized to be adversely affected in spaceflight and may partially explain the decreased bone health and generally poor quality of fracture healing in animal models flown in space. The incomplete understanding of hMSC behavior, as related to bone health in space, may jeopardize the success of future, long-duration manned missions; however, CTM provides a way to improve our understanding.

While CTM is a powerful tool for space biologists, the design that we've presented can only be used to simulate microgravity in 2D, i.e. one axis of rotation. Although this is not considered a major hurdle in microgravity research, as other investigators still use 2D clinostats, 3D microgravity simulation through random positioning machines may be a superior model for

203 microgravity. In order to achieve 3D clinorotation on a microscope stage-amenable platform,
204 clinochip devices would need to be significantly reduced in size. Also, a completely new type of
205 rotary joint would need to be designed to accommodate the additional axis of rotation. These
206 design limitations can also be considered for future work.

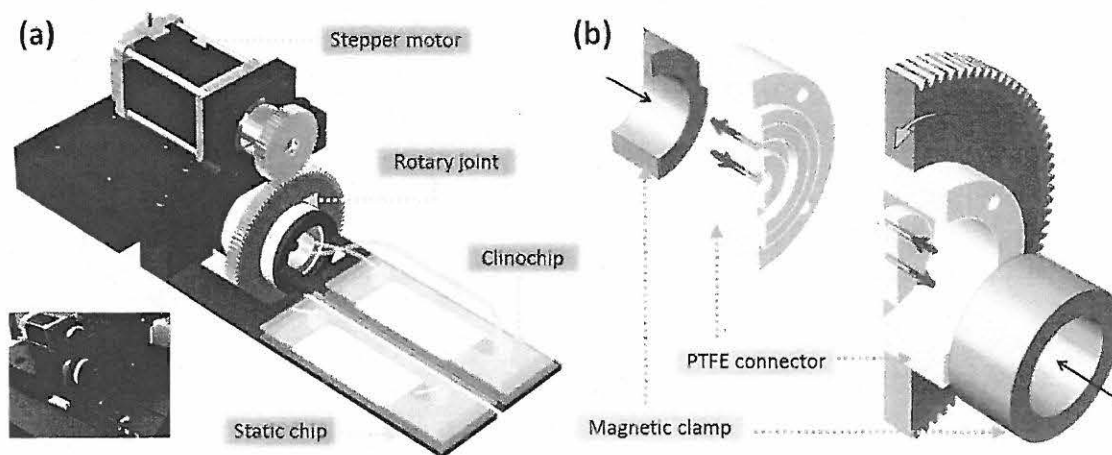


Figure 1. Microscope stage-amenable, Clinorotation Time-lapse Microscopy (CTM) system enables live-cell imaging of cells. (a) CTM components include a clinochip for simulated microgravity and static chip for a 1-g static control. (b) exploded computer model of rotary union designed to allow media perfusion into clinochips for long-term cell culture.

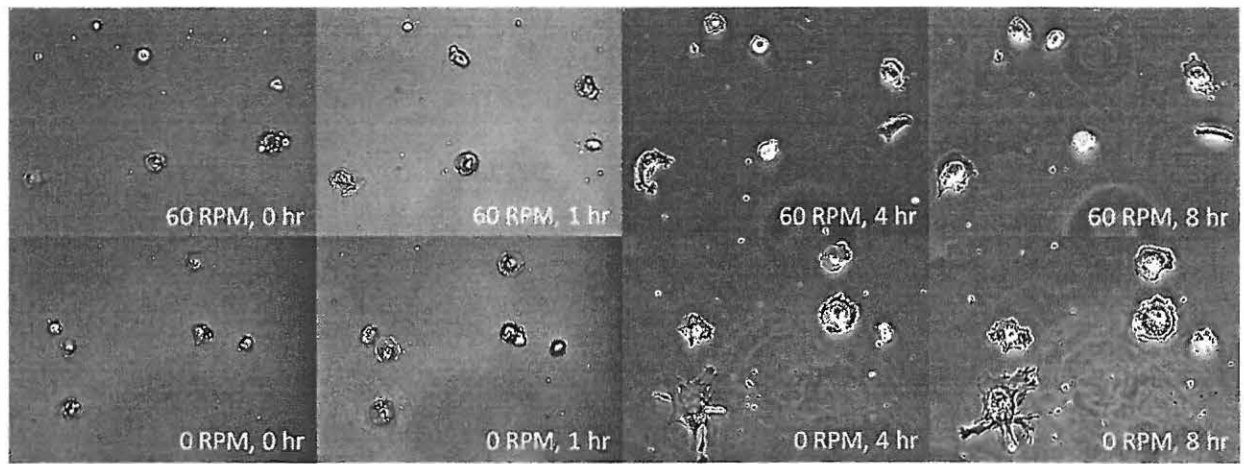


Figure 2. Time-evolution of early spreading in hMSCs imaged under DIC and phase contrast at 60 RPM clinorotation and at 0 RPM static control. Cells at 0 RPM were more spread at 4-8 hrs compared to 60 RPM.

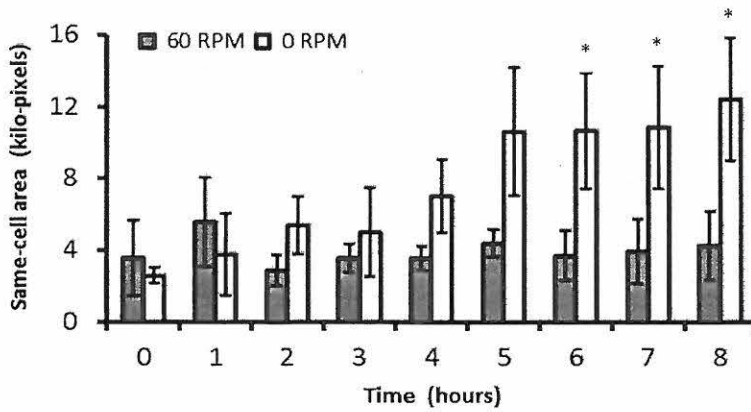
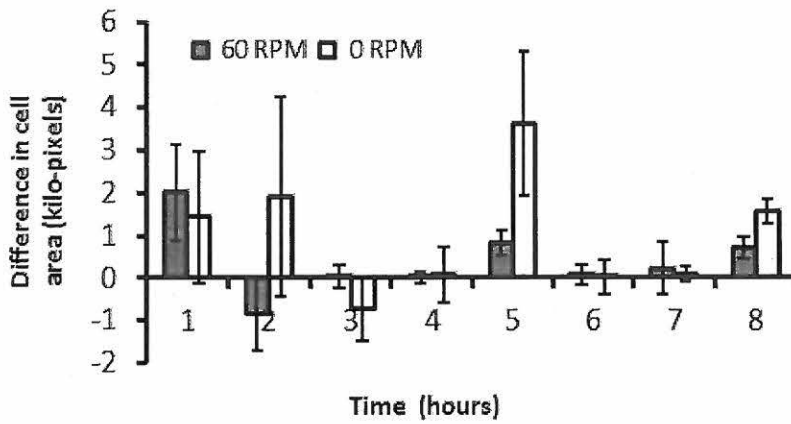


Figure 3. Mean values of same-cell areas ($n=3$) and 1 S.D. error bars. From calculated cell areas at 8 hrs (based on images from Fig 2), cells with the three median values were digitally-tagged. To eliminate outliers in cell behavior, only the tagged cells were then used to calculate areas at all remaining time points and used for comparison of means. * $p<0.05$ difference in cell area between the 60 and 0 RPM chips.



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228 **Figure 4.** Difference in cell area between current time point and previous time point ($n=3$) and
 229 1 S.D. error bars. To eliminate outliers in cell behavior, only the 3 median values of difference
 230 were used for analysis. Specimens at 0 RPM averaged 70% higher differences when compared
 231 with 60 RPM.

References

- [1] D.H. Slentz, G.A. Truske, W.E. Kraus, Effects of chronic exposure to simulated microgravity on skeletal muscle cell proliferation and differentiation, *In Vitro Cell Dev Biol Anim*, 37(3) (2010) 148-156.
- [2] K. Hirasaka, T. Nikawa, L. Yuge, I. Ishihara, A. Higashibata, N. Ishioka, A. Okubo, T. Miyashita, N. Suzue, T. Ogawa, M. Orada, K. Kishi, Clinorotation prevents differentiation of rat myoblastic L6 cells in association with reduced NF- κ B signaling, *Biochim Biophys Acta*, 1743(1) (2005) 130-140.
- [3] C. Ontiveros, L.R. McCabe, Simulated microgravity suppresses osteoblast phenotype, Runx2 levels and AP-1 transactivation, *J Cell Biochem*, 88(3) (2002) 427-437.
- [4] L. Vico, P. Collet, A. Guignandon, M.H. Lafage-Proust, T. Thomas, M. Rehailla, C. Alexandre, Effects of long-term microgravity exposure on cancellous and cortical weight-bearing bones of cosmonauts, *Lancet*, 355(9215) (2000) 1607-1611.
- [5] A.D. Leblanc, V.S. Schneider, H.J. Evans, D.A. Engelbretson, J.M. Krebs, Bone mineral loss and recovery after 17 weeks of bed rest. *J Bone Miner Res*, 5(8) (2009) 843-850.

- [6] A. LeBlanc, V. Schneider, L. Shackelford, S. West, V. Oganov, A. Bakulin, L. Voronin,
Bone mineral and lean tissue loss after long duration space flight. *J Musculoskelet Neuronal
Interact*, 1(2) (2000) 157-60.
- [7] S. Gupta, S.L. Manske, S. Judex, Increasing the number of unloading/reambulation cycles
does not adversely impact body composition and lumbar bone mineral density but reduces
tissue sensitivity. *Acta Astronaut* (2012), in press.
- [8] J.V. Sayson, A.R. Hargens, Pathophysiology of low back pain during exposure to
microgravity. *Aviat Space Environ Med*, 79(4) (2008) 365-373.
- [9] S.L. Johnston, M.R. Campbell, R. Scheuring, A.H. Feiveson, Risk of herniated nucleus
pulposus among US astronauts. *Aviat Space Environ Med*, 81(6) (2010) 566-574.
- [10] R.A. Scheuring, C.H. Mathers, J.A. Jones, M.L. Wear, Musculoskeletal injuries and minor
trauma in space: incidence and injury mechanisms in US astronauts. *Aviat Space Environ
Med*, 80(2) (2009) 117-124.
- [11] D.M. Klaus, Clinostats and bioreactors. *Gravit Space Biol Bull*, 14(2) (2007) 55-64.
- [12] M. Cogli, The fast rotating clinostat: a history of its use in gravitational biology and a
comparison of ground-based and flight experiment results, *Gravit Space Biol Bull*, 5(2)
(1992) 59-67.

[13] J.J. van Loon, Some history and use of the random positioning machine, RPM, in gravity related research, Adv Space Res, 39(7) (2007) 1161-1165.

[14] European Space Agency. DLR – Clinostats, centrifuges, RPM. Human spaceflight research. Retrieved December 20, 2012, from [http://www.esa.int/Our_Activities/Human_Spaceflight/Human_Spaceflight_Research/DLR_-](http://www.esa.int/Our_Activities/Human_Spaceflight/Human_Spaceflight_Research/DLR_-_Clinostats_Centrifuges_RPM)
- _Clinostats_Centrifuges_RPM

[15] C. Pache, J. Kühn, K. Westphal, M.F. Toy, J. Parent, O. Büchi, A. Franco-Obregón, C. Depeursinge, M. Egli, Digital holographic microscopy real-time monitoring of cytoarchitectural alterations during simulated microgravity, J Biomed Opt, 15(2) (2010) 026021-026021.

[16] M.F. Toy, S. Richard, J. Kühn, A. Franco-Obregón, M. Egli, C. Depeursinge, Enhanced robustness digital holographic microscopy for demanding environment of space biology, Biomed Opt Express, 3(2) (2012) 313-326.

[17] H. Finkelstein, N. Dvorochkin, R. Yousuf, R.K. Globus, E.A. Almeida, Spaceflight Reduces the Tissue Regenerative Potential of Stem Cells by Decreasing Proliferation and Increasing Early Differentiation. Stem Cells Biology Poster Session. 50th Annual Meeting of the American Society for Cell Biology, Philadelphia, PA, December 11-15, 2010.

299 [18]M. Zayzafoon, W.E. Gathings, J.M. McDonald, Modeled microgravity inhibits osteogenic
300 differentiation of human mesenchymal stem cells and increases adipogenesis.
301 Endocrinology, 145(5) (2004) 2421-2432.

302

303 [19]V.E. Meyers, M. Zayzafoon, J.T. Douglas, J.M. McDonald, RhoA and cytoskeletal
304 disruption mediate reduced osteoblastogenesis and enhanced adipogenesis of human
305 mesenchymal stem cells in modeled microgravity. J Bone Miner Res, 20(10) (2005) 1858-
306 1866.